

REMARKS

On page 2 of the response filed on April 2, 2009, applicants cited US Patent **7,446,188**, as stating:

"The gene can be maintained episomally or the gene can be integrated into the chromosome."

This cite actually stems from column 17 (l. 54-55) of US Patent **7,410,788**, issued August 12, 2008 which was also examined in Art Unit 1638 (highlighted excerpt attached).

U.S. Patent **7,446,188**, issued on August 14, 2008, which was examined in Art Unit 1638, cites in column 56 (l. 10-13) (highlighted excerpt attached):

"In some embodiments, the vector is maintained episomally. In other embodiments, the vector is integrated into the genome."

Applicants respectfully request consideration of both of these citations and entry of this supplemental response which only supplements and does not replace the response filed on April 2, 2009.

The Commissioner is authorized to charge any fee deficiencies or overpayments to undersign's deposit account 50-3135.

Respectfully submitted,

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5,824,877; and 4,940,838; all of which are incorporated herein by reference). Construction of recombinant Ti and Ri plasmids in general follows methods typically used with the more common bacterial vectors, such as pBR322. Additional use can be made of accessory genetic elements sometimes found with the native plasmids and sometimes constructed from foreign sequences. These may include but are not limited to structural genes for antibiotic resistance as selection genes.

There are two systems of recombinant Ti and Ri plasmid vector systems now in use. The first system is called the "cointegrate" system. In this system, the shuttle vector containing the gene of interest is inserted by genetic recombination into a non-oncogenic Ti plasmid that contains both the cis-acting and trans-acting elements required for plant transformation as, for example, in the pMLJ1 shuttle vector and the non-oncogenic Ti plasmid pGV3850. The second system is called the "binary" system in which two plasmids are used; the gene of interest is inserted into a shuttle vector containing the cis-acting elements required for plant transformation. The other necessary functions are provided in trans by the non-oncogenic Ti plasmid as exemplified by the pBIN19 shuttle vector and the non-oncogenic Ti plasmid PAL404. Some of these vectors are commercially available.

In other embodiments of the invention, the nucleic acid sequence of interest is targeted to a particular locus on the plant genome. Site-directed integration of the nucleic acid sequence of interest into the plant cell genome may be achieved by, for example, homologous recombination using *Agrobacterium*-derived sequences. Generally, plant cells are incubated with a strain of *Agrobacterium* which contains a targeting vector in which sequences that are homologous to a DNA sequence inside the target locus are flanked by *Agrobacterium* transfer-DNA 1-DNA sequences, as previously described (U.S. Pat. No. 5,501,967). One of skill in the art knows that homologous recombination may be achieved using targeting vectors which contain sequences that are homologous to any part of the targeted plant gene, whether belonging to the regulatory elements of the gene, or the coding regions of the gene. Homologous recombination may be achieved at any region of a plant gene so long as the nucleic acid sequence of regions flanking the site to be targeted is known.

In yet other embodiments, the nucleic acids of the present invention is utilized to construct vectors derived from plant (+) RNA viruses (for example, brome mosaic virus, tobacco mosaic virus, alfalfa mosaic virus, cucumber mosaic virus, tomato mosaic virus, and combinations and hybrids thereof). Generally, the inserted plant CPA-FAS polynucleotide of the present invention can be expressed from these vectors as a fusion protein (for example, coat protein fusion protein) or from its own subgenomic promoter or other promoter. Methods for the construction and use of such viruses are described in U.S. Pat. Nos. 5,846,795; 5,500,360; 5,173,410; 5,965,794; 5,977,438; and 5,866,785, all of which are incorporated herein by reference.

In some embodiments of the present invention, where the nucleic acid sequence of interest is introduced directly into a plant. One vector useful for direct gene transfer techniques in combination with selection by the herbicide Basta (or phosphinothricin) is a modified version of the plasmid pCIB246, with a CaMV 35S promoter in operational fusion to the *E. coli* GUS gene and the CaMV 35S transcriptional terminator (WO 93/07278).

c. Transformation Techniques

Once a nucleic acid sequence encoding a plant CPA-FAS is operatively linked to an appropriate promoter and inserted into a suitable vector for the particular transformation technique utilized (for example, one of the vectors described above), the recombinant DNA described above can be introduced into the plant cell in a number of art-recognized ways. Those skilled in the art will appreciate that the choice of method might depend on the type of plant targeted for transformation. In some embodiments, the vector is maintained epically. In other embodiments, the vector is integrated into the genome.

In some embodiments, direct transformation in the plastid genome is used to introduce the vector into the plant cell (See for example, U.S. Pat. Nos. 5,451,513; 5,545,817; 5,545,818; PCT application WO 95/16783). The basic technique for chloroplast transformation involves introducing regions of cloned plastid DNA flanking a selectable marker together with the nucleic acid encoding the RNA sequences of interest into a suitable target tissue (for example, using biolistics or protoplast transformation with calcium chloride or PEG). The 1 to 1.5 kb flanking regions, termed targeting sequences, facilitate homologous recombination with the plastid genome and thus allow the replacement or modification of specific regions of the plastome. Initially, point mutations in the chloroplast 16S rRNA and rps12 genes conferring resistance to spectinomycin and/or streptomycin are utilized as selectable markers for transformation (Svab et al. (1990) PNAS, 87:8526; Staub and Maliga, (1992) Plant Cell, 4:39). The presence of cloning sites between these markers allowed creation of a plastid targeting vector introduction of foreign DNA molecules (Staub and Maliga (1993) EMBO J., 12:601). Substantial increases in transformation frequency are obtained by replacement of the recessive rRNA or r-protein antibiotic resistance genes with a dominant selectable marker, the bacterial aadA gene encoding the spectinomycin-detoxifying enzyme aminoglycoside-3'-adenyltransferase (Svab and Maliga (1993) PNAS, 90:913). Other selectable markers useful for plastid transformation are known in the art and encompassed within the scope of the present invention. Plants homoplasmic for plastid genomes containing the two nucleic acid sequences separated by a promoter of the present invention are obtained, and are preferentially capable of high expression of the RNAs encoded by the DNA molecule.

In other embodiments, vectors useful in the practice of the present invention are microinjected directly into plant cells by use of micropipettes to mechanically transfer the recombinant DNA (Crossway (1985) Mol. Gen. Genet, 202:179). In still other embodiments, the vector is transferred into the plant cell by using polyethylene glycol (Krens et al. (1982) Nature, 296:72; Crossway et al. (1986) BioTechniques, 4:320); fusion of protoplasts with other entities, either minicells, cells, lysosomes or other fusible lipid-surfaced bodies (Fraley et al. (1982) Proc. Natl. Acad. Sci., USA, 79:1859); protoplast transformation (EP 0 292 435); direct gene transfer (Paszowski et al. (1984) EMBO J., 3:2717; Hayashimoto et al. (1990) Plant Physiol. 93:857).

In still further embodiments, the vector may also be introduced into the plant cells by electroporation. (Fromm, et al. (1985) Pro. Natl Acad. Sci. USA 82:5824; Riggs et al. (1986) Proc. Natl. Acad. Sci. USA 83:5602). In this technique, plant protoplasts are electroporated in the presence of plasmids containing the gene construct. Electrical impulses of high field strength reversibly permeabilize biomembranes allowing the introduction of the plasmids. Electroporated plant protoplasts reform the cell wall, divide, and form plant callus.

cysteine (amino acid 47) that is located in the catalytic site of the enzyme. A nucleic acid encoding this mutated form of AhpC is set forth in SEQ ID NO: 23, and the amino acid encoded therefrom is set forth in SEQ ID NO: 24. The wild type nucleic acid and amino acids of AhpC are set forth in SEQ ID NO: 21 and 22, respectively, and correspond to GenBank Accessions Nos. D13187 (Feb. 3, 1998) and BAA02485 (Feb. 3, 1998), respectively. As further described in the Examples, this mutation essentially destroys the peroxidase activity of the enzyme. As further shown herein, the presence of mutated AhpC (referred to as AhpC*) provides growth enhancing capability to host cells only in the presence of AhpF and of a functional glutaredoxin system. Thus, it is likely that AhpC* enhances growth by reducing oxidized glutaredoxin I or glutathione. Generally, it is believed that the AhpC* increases the reducing capacity to the cytoplasm sufficient to allow growth.

Accordingly, growth of host cells can also be improved by introducing a modification in the host cell which increases the reducing capacity of its cytoplasm. The modification can be a mutation in a gene of the host cell, e.g., a mutation which increases the reducing potential of an enzyme, or which reduces the oxidizing potential of an enzyme. A preferred modification is a mutation in the AhpC gene, e.g., a mutation in its catalytic domain. An even more preferred mutation is one that occurs in the TCT triplet repeat, such as the insertion of a TCT triplet, as shown in FIG. 8A. A preferred mutant AhpC has the amino acid sequence set forth in SEQ ID NO: 24. Other mutations can also be made to AhpC, provided that the mutation improves the growth of the cells. Identification of other mutations, e.g., in AhpC, that have a growth improving activity can be identified, e.g., by introducing random mutations in a host cell, e.g., one having mutations in *trxB* and in *gor*, and selecting for those having enhanced growth. The mere culture of such mutated cells will result in an enriched population of cells having growth inducing mutations, which can then be identified. Random mutations can be introduced and identified according to methods well known in the art of prokaryotic genetics.

As opposed to introducing a mutation in a particular gene to induce growth, one may also downregulate the expression of the gene by any of a variety of methods, including antisense expression or the contacting the cell with an agent that reduces transcription of the gene.

Alternatively, the modification of host cells can be the introduction into the host cell of a gene which enhances growth or stimulating the expression of a gene enhancing growth in the host cell. For example, a host cell can be modified by the introduction into the cell of a gene encoding a protein which increases the reducing capacity of the cytoplasm. In a preferred embodiment, the gene is a reductase. In an even more preferred embodiment, the gene encodes AhpC*. The gene can be maintained episomally or the gene can be integrated into the chromosome. It may be desirable, in certain circumstances to reduce or eliminate the amount of the corresponding protein of the growth inducing gene. In the case in which a gene encoding AhpC* is introduced into a cell, and optionally overexpressed, it is not necessary to reduce expression of the wild type gene encoding AhpC, since it has been shown herein that AhpC* is dominant.

In view of the strong conservation of the AhpC genes across species (see, e.g., FIG. 8B), host cells other than *E. coli* can be modified in a similar fashion to improve their growth potential. For example, a host cell can be modified by introducing a gene encoding a mutated AhpC protein, such as one having a mutation in the repeated triplet region.

It is likely that the reason the *trxB,gor* and *trxB,gshA* strains do not grow is that they do not have sufficient reducing power to maintain the essential enzyme ribonucleotide reductase in the reduced, active state. Accordingly, another class of suppressors that may restore growth to these strains is one in which one (or more) of the several ribonucleotide reductase genes on the *E. coli* chromosome is altered by mutation so that it no longer needs the thioredoxin or glutathione/glutaredoxin pathways as a source of reducing power. It would obtain its electrons from one of the other possible sources in the cytoplasm. Such suppressor strains may, in addition, be even more efficient at disulfide bond formation than the strains having a mutation in *ahpC* because, in contrast to the likely consequence of the *ahpC* mutation, these suppressor mutations do not generate any new reducing power. The cytoplasm may well be more oxidizing vis-a-vis disulfide bonds than FA113.

Modification of Host Cells by the Addition of Genes Encoding Catalysts of Disulfide Bond Formation and/or Isomerization

As shown in the Examples, proper folding of polypeptides comprising numerous disulfide bonds expressed in host cells was increased by cotransformation of the host cell with a catalyst of disulfide bond formation and/or a catalyst of disulfide bond isomerization. Thus, generally the invention provides host cells which are modified to over-express or increase the activity of one or more catalyst(s) of disulfide bond formation and/or isomerization.

In a preferred embodiment, a catalyst of disulfide bond formation is an enzyme which facilitates, or increases the speed of, disulfide bond formation. Generally, a catalyst of disulfide bond formation will have the following characteristics: it is able to accumulate in oxidized form in the cytoplasm, and the oxidized form of the protein catalyst is efficient at transferring its disulfide to a substrate protein. Accordingly, since a catalyst of disulfide bond formation must be in oxidized form in the cytoplasm to be active, the catalyst will generally have a low redox potential, e.g., in the range of the redox potential of the thioredoxins and glutaredoxins. Thus, catalysts of disulfide bond formation will preferably have a redox potential of at most about -270 mV, preferably at most about -260 mV, at most about -250 mV, at most about -240 mV, at most about -230 mV, at most about -220 mV, at most about -210 mV, at most about -200 mV, or at most about -190 mV. Other preferred catalysts have a redox potential in the range of about -260 to -190 mV, more preferably, of about -230 to -190 mV, and even more preferably of about -210 to -190 mV. However, catalysts of disulfide bond formation can also have a redox potential outside of these ranges, provided that the enzyme is capable of catalyzing disulfide bond formation, as can be shown in *in vitro* or *in vivo* assays, as further set forth herein.

Catalysts of disulfide bond isomerization are enzymes which are capable to form disulfide bonds, but which are also capable of shuffling disulfide bonds. Generally, catalysts of disulfide bond isomerization will be in a reduced state in the cytoplasm, so that they are capable of invading incorrectly formed disulfide bonds. Accordingly, an isomerase will generally have a higher redox potential than a catalyst of disulfide bond formation. Preferred isomerases have a redox potential of at most about -200 mV, at most about -190 mV, at most about -180 mV, preferably at most about -170 mV, preferably at most about -160 mV, and most preferably at most about -150 mV. However, an isomerase can also have a redox potential outside of these ranges, provided that the enzyme is